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HUMAN INDUCED PLURIPOTENT STEM CELLS IN REGENERATIVE MEDICINE

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Human Induced Pluripotent Stem Cells in Regenerative Medicine

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Human induced pluripotent (iPS) cells offer a theoretically inexhaustible cell source for the derivation of any cell type and are a promising tool in regenerative medicine. Cells can be derived from the recipients own tissue or from pre-selected HLA-matched donors. Certain disease relevant cell types derived from iPS cells can be cryopreserved, distributed world-wide and made available on demand. These factors taken together indicates iPS cells could potentially revolutionize medicine.

Technological maturation has been fast paced with rapid advances in derivation and culture techniques in the last decade, creating cells of sufficient quality to motivate in human use. The first clinical trials performed with iPS cell derived grafts have so far been conducted without indications of adverse events. Pioneering applications are now being evaluated in humans. Meanwhile, academia and industry alike are taking strides to industrialize cell manufacture in anticipation of commercial applications. Strategies utilizing genome editing are opening up entirely new venues for future applications.

However, challenges does not solely lay in developing efficacious cell therapies. Pioneering cell therapy products have so far been exceptionally costly. Finding strategies minimize cost may be as an important as proving efficacy if any novel cell therapies are ever going to benefit society.

Regulations dictating the use of iPS cell derived cell therapies remains unspecific yet demanding and insufficiently harmonized internationally. Efforts are ongoing to coordinate in-between regulatory authorities and more defined criteria for manufacture and regulatory approval is slowly emerging.

It has now been little over 10 years since the derivation of the first human iPS cell lines. A decade which has seen many barriers overcome. The upcoming decade is likely to be even more exciting and challenging, as therapies will need, not only to be consistently proven safe, but also efficacious and commercially viable.

LIST OF SCIENTIFIC PAPERS

I. **Uhlin, E.** Rönnholm, H. Day, K. Kele, M. Tammimies, K. Bölte, S. Falk, A.
Derivation of Human iPS Cell Lines from Monozygotic Twins in Defined and Xeno Free Conditions.
Stem Cell Research. doi.org/10.1016/j.scr.2016.12.006

II. **Uhlin**, **E.** Marin Navarro, A. Rönnholm, H. Day, K. Kele. Falk, A.
Integration Free Derivation of Human Induced Pluripotent Stem Cells Using Laminin 521 Matrix.
Journal of Visualized Experiments. DOI: 10.3791/56146

III. **Uhlin**, **E.** Kele, M. Rönnholm, H. Day, K. Ulfenborg, H. Anderlid, B. Falk, A.
Derivation of a GMP-Compliant Human iPS Cell Line "KICRi001-A" from Dermal Fibroblasts by RNA-Mediated Reprogramming.
Submitted manuscript, In revision.

IV. **Uhlin**, **E.** Kele, M. Winblad, N. Baqué–Vidal, L. Petrus-Reurer, S. Bengtsson, M. Lanner, F. Falk, A.
A Strategy for the Creation of a Clinical Grade iPS Cell Bank Covering a Majority of the World Population by CRISPR-Cas9 Genome Editing.
Manuscript.

V. **Uhlin**, **E.** Morse, R. Kele, M. Holmberg, L. Xu, L. Moslem, M. Sundström, E. Falk, A.
Pre-Clinical Evaluation of Clinically Relevant iPS Cell Derived Neuroepithelial Stem Cells as an Off-the-Shelf Cell Therapy for Spinal Cord Injury.
Manuscript.

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Calvo-Garrido, J. Maffezzini, C. Schober, F. Clemente, P. **Uhlin**, **E.** Kele, M. Stranneheim, H. Lesko, N. Bruhn, H. Svenningsson, P. Falk, A. Wedell, A. Freyer, C. Wredenberg, A.
SQSTM1/p62-Directed Metabolic Reprogramming Is Essential for Normal Neurodifferentiation.
Stem Cell Reports. doi.org/10.1016/j.stemcr.2019.01.023

II. Lam, M. Moslem, M. Bryois, J, Pronk, R. **Uhlin**, **E.** Dehnisch Ellström, I. Laan, L. Olive, J. Morse, R. Rönnholm, H. Louhivuori, L. Korol, S. Dahl, N. Uhlén, P. Anderlid, B. Kele, M. Sullivan, P. Falk, A.
Single Cell Analysis of Autism Patient with Bi-Allelic NRXN1-Alpha Deletion Reveals Skewed Fate Choice in Neural Progenitors and Impaired Neuronal Functionality.
Experimental Cell Research. doi.org/10.1016/j.yexcr.2019.06.014

CONTENTS

LIST OF ABBREVIATIONS

iPS Cells	Human induced pluripotent stem cells
SCNT	Somatic cell nuclear transfer
OKSM	OCT4, KLF4, SOX2 and c-MYC
ES cells	Human embryonic stem cell
PS cells	Pluripotent stem cells
MEF cells	Mouse embryonic fibroblasts cells
SCI	Spinal cord injury
ATMP	Advanced therapy medicinal product
HLA	Human leukocyte antigen
IP	Intellectual property
PBMC	Peripheral blood mononuclear cells
FBS	Fetal bovine serum
EMA	European medicines agency
FDA	Food and drug administration
ISSCR	International society for stem cells research
ICH-GCP	The international conference on harmonization of good clinical practice
GLP	Good laboratory practice
GMP	Good manufacturing practice
OPC	Oligodendrocyte progenitor cells
MSC	Mesenchymal stem cells
NS cells	Neural stem cells
NES cells	Neuroepithelial stem cells
RPE cells	Retinal pigment epithelium cells
CIITA	Class II trans activator
B2M	Beta-2-Microglobulin
NK-cells	Natural killer cells
CAR-T cells	Chimeric antigen receptor T-cell
CNS	Central nervous system

1 REGENERATIVE MEDICINE

1.1 REGENERATIVE MEDICINE THROUGH THE AGES

The field of regenerative medicine can seem like science fiction. The fundamental concept behind it is to repair, replace or regenerate cells, organs or tissues to restore functions[4]. Certain tissues of the adult human body are only capable of very limited tissue regeneration. This is in stark contrast to early development were totipotent and pluripotent stem cells has the potential to proliferate and differentiate into all tissues that make up the body. A loss of stemness occur as cells differentiate and specialize. If adult cells could be reverted to earlier developmental stage, the regenerative potential of stem cells could be unleashed to repair damages injuries the adult physiology are unable to handle.

Stem cell therapy is not an entirely novel concept, transplantations of adult hematopoietic stem cells derived from bone marrow has been in clinical use since the 1960s. Rapid developments in the field in the 1990s enabled the culture and derivation of multiple new stem cell types with therapeutic potential[5]. Transplantation of adult stem-cells-like mesenchymal stem cells was first clinically evaluated in 1995[6], although transplantation of these cells are being considered in a broad spectrum of condition today, the limited differentiation potential of adult stem cells constrain their regenerative abilities[7]. Transplantations trails using fetal-derived progenitor cells was pioneered with mixed results in Parkinson's disease, age related macular degeneration, spinal cord injury and heart failure, to mention a few[8-10]. These studies have come to serve as proof-of-concept in regenerative medicine but the limited quantity, and quality of fetal tissue available meant that large-scale therapies are not feasible[11].

1.2 PLURIPOTENT STEM CELLS

The capture of the first human embryonic stem (ES) cell line was in 1998[12] drastically widened the potential of cell therapies bringing with it a vision of seemingly limitless potential. ES cells are able to generating any tissue of the adult human body on theoretically limitless scale[12]. Several ES cell-derived therapies have since progressed to clinical trials[13]. However, the use of ES cells has and is still the topic of harsh debate. Ethical concerns mainly focusing on the origin the of ES cells. Pluripotent cells can also be derived by somatic cell nuclear transfer (SCNT) but is technically complicated[14] and also raises ethical dilemmas concerning the destruction of viable human embryos[15]. Pathogenic stem cells, chemically induced uniparental zygotes unable to develop into a viable embryo but with clonal self-renewal capacity partly forgo those argumentations. Pathogenic stem cells display unique epigenetic imprinting which limits their linage specification ability and application[16].

A breakthrough came in 2006 with the advent of a new type of human pluripotent cell type, the so called induced pluripotent stem (iPS) cells. These cells circumvent the ethical and legal concerns of ES cells altogether. The discovery was made by a team of Japanese scientists exploring the possibility to reactivate the pluripotency transcriptional pathways in adult

somatic cells and by doing so creating an artificial embryonic like stem cell[17]. Effectively turning back the developmental machinery of adult cell to a stage a few days after fertilization.

1.3 THE REPROGRAMMING PROCESS

1.3.1 Reversing the Order of Development Determined by 3,5 Billion Years of Evolution

Genes code for protein, proteins shape life in the form of cells and cells form complex organisms that procreate, age and die. The cycle of life, constantly moving in a determined direction obeying the laws written and formed by small forces acting over uncomprehensive long times. The rules are ancient, but science has previously enabled us to break such laws of nature.

Our genes are inherited through our DNA which serves as a blueprint, coding mostly for protein production. Proteins is the incredibly versatile molecules of life, tools through which our cells form incredibly diverse tissues. Proteins are differently expressed in different tissues, giving rise to a plethora of different cell types in our bodies. Either selective loss of DNA occurs during development or intricate processes, invisible to the naked eye, irreversibly control how genes are expressed.

Decades before the molecular structure of our DNA had been determined it was proposed that the presence of certain genes alone might not be solely responsible for the functions of an organism and hence the field of epigenetics was born[18]. The hypothesized elements of epigenetics were working on a step above the genome, modulating the expression and thereby allowing the functions of cells to diverge. The process was conceptualized by Waddington et al.[1] as a marble rolling down a hill getting caught in valleys as it irreversibly rolls down the hill of development but never up (Figure 1)[1]. At the summit of this model, is the zygote, the first cell of any individual, imbued with the potential to create entire organisms in all their diversity. Each intersection, represent a fate-choice and a restriction in the potency of the marble.

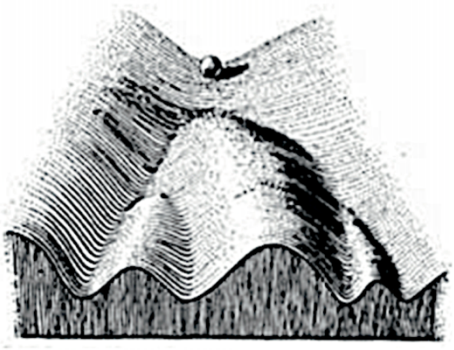

Figure 1. Epigenetic landscape proposed by Waddington et al.[1] Adapted from S.Bhattacharya et al. 2011[3]

Cancers gave some indication that it was perhaps possible to partly change the predetermined fate of cells but it was not until John Gurdon pioneered his landmark experiments on SCNT that the irreversibility of cellular differentiation by experimental means was disproven. The nuclei from an oocyte from a tadpole (*Rana pipiens*) was replaced with an intestinal epithelial cell nuclei resulting in the development and birth of an new individual. This experiment suggested that even mature cells retain the complete genetic information suggesting that

certain factors excise firm control of gene expression and thereby dictating cell state.[15, 19, 20]. This concept was proven similarly in mammals in 1996 with the birth of Dolly the sheep[21].

1.3.2 To Master the Genome

The knowledge that even adult cell retains a full genome and uncovering the prospect of reverting adult cells to earlier developmental origins had an enormous implication for medicine. If the epigenetics could be controlled, tissues otherwise incapable of regeneration could be reverted to an earlier stage of development were cells were potent to form the tissue. The epigenome operates is on several levels, through diverse actions, acting both pre- and post-transcriptionally. Mechanisms include control of chromatin state, DNA methylation and non-coding RNAs[22] A stride towards controlling cell fate was taking in 1987 when the programing of fibroblast cells into myoblasts by introducing specific genes via retroviral vectors, later known as transcriptions factors[23]. Specific combinations of transcription factors regulate batteries of genes which determine and maintain cell fate[24]. The knowledge that transcription factors held the potential to influence fate-switching in cells, encouraged the study of transcription factors particularly expressed in oocytes and ES cell maintenance. It was by doing this, Yamanaka et. al managed cell reprogramming by a defined set of ectopically expressed reprogramming factors. Out of many, four factors were identified as sufficient to reverse the otherwise irreversible flow of time[17]. Although other factor combinations has since proven possible[25] the Yamanaka factors remain in use to this day[26].

1.3.3 Mechanisms of Reprograming

The mechanisms of reprogramming using the Yamanaka factors OCT4, KLF4, SOX2 and c-MYC (OKSM) has been described to occur in two transcriptional waves[27]. First, a stochastic wave, were c-MYC is thought to be responsible for repression of somatic genes and upregulation of proliferation. KLF4 also indicated to play a role in the repression of somatic factors in this early state. Typical for the early stage of the reprogramming process is dedifferentiation, increase in proliferation, loss of somatic gene expression, and upregulation of proliferation associated genes. The effects are highly heterogenous on the cells and only a few will actually proceed to the second stage[28]. The second wave of transcriptional activity follow a more deterministic sequence of events which is dependent on OCT4, SOX2 and KLF4 triggering the expression of the pluripotency genes[28, 29]. Only a rare few cells will successfully pass through these sequences of events and manage to switch on and endogenously maintain the pluripotency circuitry[28]. Regardless of the factors used for reprogramming the end result is similar. This indicates that the factors used for reprograming either serve similar functions in reprogramming or trigger transcription of a series of other factors essential for pluripotency[30].

The process of reprograming described above deviates from that observed in SCNT which is rapid and more deterministic, also in the early phase[31]. The more controlled environment and reprogramming factors at physiological levels in the oocyte is thought to contribute. This indicates that current reprogramming schemes could potentially yet be improved.

1.3.4 A Golden Standard for Perfecting Reprogramming

Perhaps not all iPS cells are created equal. Fortunately, there are pluripotent stem cells from embryonic sources which long has served as a golden standard. ES cells are derived from the inner cell mass of the blastocyst around day 5-6 after fertilization[12]. The cells that are destined to become iPS cells go far beyond the day 5-6 days, and are derived from post-natal tissue, possibly after several decades of life before being reprogrammed back an ES-like state. A question that has preoccupied the field for years is if this difference in cell source is reflected in meaningful functional differences.

1.3.5 Epigenetic Memory

Beyond the apparent similarity in form and function between ES and iPS cells, early reports indicated small but potentially significant differences observed in-between ES cells and iPS. Slight differences in gene expression profiles[32], differences in methylation patterns [33] and epigenetic memory retained from tissue of origin[34] was reported. The epigenetic memory was indicated to give preferential differentiation into tissue types remising of cell type origin[35] however this epigenetic memory could be lost with modest passaging of iPS cells[34]. With as little as ten passages the epigenetic memory was near eliminated and with sixteen passages cells were indistinguishable based on origin[35]. Later studies comparing isogenic iPS cells derived from different tissue sources found that the variation in differentiation propensity is rather due to donor dependent variations in expression and methylation rather than from the tissue of origin[36, 37].

A recent study of over 700 iPS cell lines from 301 donors identified the donor as the biggest source of variance, accounting for 46% of variation, 23% was attributed copy number variations, 26% ascribed to culture condition, 2% to passage number and 2% gender[38]. Although most but not all iPS cell lines can be distinguished from ES cell lines based on variability in transcription, the individual difference between any pluripotent lines is larger than variability in-between ES cells and iPS cells[39]. It may therefore be reasonable to put more focus on the variability in-between cell lines rather than the source of the pluripotent stem cells[36, 37, 39-41].

1.3.6 Differentiation Bias

Pluripotent stem (PS) cells are theoretically capable of deriving most cell types if not all, however they do display potential bias towards certain germ layers[42]. This does not mean certain lines cannot differentiate into certain cell types, but certain lines may do so at lower efficiencies. The differences is due to discrete variations in DNA methylation patterns and gene expression levels in-between cell lines, which is a phenomenon observed in both ES and iPS cell lines[43]. As we have discussed this differences is primary donor dependent rather than occurring from tissue of origin[36] but remain stable over passages[44]. Assays has been developed to predict the differentiation bias towards germ layers[43].

1.3.7 Different States of Pluripotency

Perplexing differences was observed in-between mouse ES cells[45, 46] and human ES cells[12]. Although both cell types are derived from cells of the blastocyst, key differences could not be ignored and the possibility of multiple pluripotent states was proposed[47] Mouse PS cells grow in dome like shapes, female cells have two active X chromosomes and maintenance of pluripotency depend on LIF and BMP signaling instead of FGF/Activin in human pluripotent cells[48]. The capture of human Naïve pluripotent stem cells was reported in 2013 and provided a pluripotent state in humans resembling that observed in mice[49]. Naïve pluripotent cells have a higher degree of clonogenicity, higher proliferation rates, low degree of differentiation bias and are more permissive to genome editing[50-52]. In this text, the first discovered state, the primed state is discussed unless otherwise stated.

Naïve cells probably closest resemble the cells of the preimplantation epiblast[53] similar to those from day 6-7 post fertilization while primed cells more likely resemble day 9-12 in the post implantation period (Figure 2). Determining the precise developmental equivalent is hindered by ethical considerations against studying human embryos[48]. The derivation of primordial germ cells is much higher with Naïve cells but seems at best very limited by primed pluripotent stem cells[54]. Naïve cells also allow the study of certain features of the preimplantation embryo[53]. With the right culture condition, primed cells can be reverted to a naïve state and vice versa without the use of transgenes.[55] Both conversion protocols and direct to naïve reprogramming have so far depended on culture on mouse embryonic fibroblasts (MEF)[51, 53, 56]. Although Naïve cells display more homogeneous differentiation to germ layers then primed cell lines their ability to differentiate to terminal functional cell types seems limited[56, 57] this in combined with the xenogeneic derivation is currently hampering their utility for regenerative medicine protocols[56]. However, one recent study have reported mature differentiation of cell types from naïve cells with adapted protocols[51].

The possibility of even further pluripotent states has been reported. Extended pluripotent stem cells with a propensity to also differentiate into extraembryonic tissues[58]. The derivation of these cells is somewhat controversial[53] since they more closely adhere to the definition of totipotency. However, their lack of autonomous developmental potential place them in a gray zone between totipotent and pluripotent.

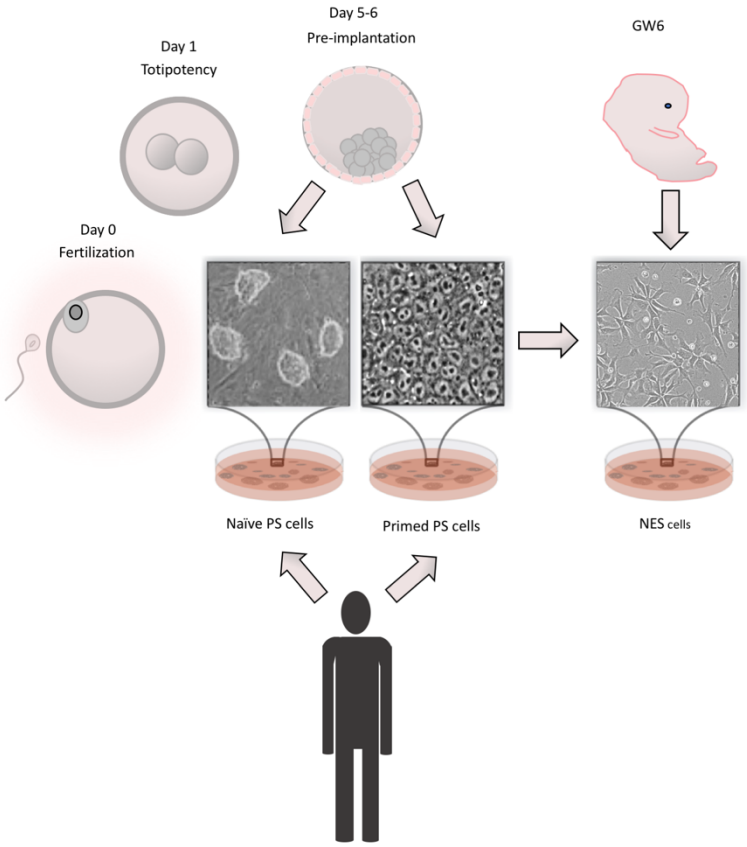

Figure 2. Early human development and derivation of pluripotent and fetal cells. The fusion of oocyte and spermatozoa result in the totipotent zygote. Both Naïve and primed ES cells are derived from the inner cell mass of the pre-implantation blastocyst. However, both most likely resemble later stages of development, Naïve around d6-7 and primed 9-12 days post fertilization. Fetal derived cells can be derived at later stages. Neuroepithelial stem (NES) are derived around Gestational week (GW) 5-7. Both Naïve and primed iPS cells can also be derived by reprogramming of adult tissues. Pluripotent stem cells can be differentiated to fetal cells such as NES cells. Their vast proliferative potential of pluripotent cells means theoretically unlimited amounts can be derived. Naive pluripotent stem cells from Theunissen et al 2014[59], adapted from Uhlin, Marin Navarro et al, 2017[2].

2 MANUFACTURE OF INDUCED PLURIPOTENT STEM CELLS

Since the derivation of the first human iPS cell lines in 2007 the progress in the field has been rapid[25, 60]. iPS cells has proven its worth in diverse fields with applications ranging from disease modelling[61-63], drug discovery[64] and regenerative medicine[65]. The use of iPS cells in the regenerative medicine has been lagging behind other applications, awaiting further developments in culture systems, vector technology, maturation of regulatory consistency and sufficient commercial interests to warrant financial feasibility of projects.

At the time of writing, at least four clinical trials has transplanted iPS cell derived grafts, in macular degeneration[13, 65], Parkinson's disease[66], graft vs host disease[67] and cancer[68]. However, a torrent of novel phase I trials are schedule to commence imminently exploring treatments of spinal cord injury (SCI)[69], Parkinson's disease[69, 70], diabetes and heart disease[71]. Trials using ES cell-derived tissues have progressed further with ongoing trials including SCI, diabetes type I[72] and heart failure[13, 73].

The reason behind the sharp rise in planned iPS cell-derived cell therapies is not coincidental. Two important hurdles have been holding clinical translation back. The first obstacle is technological maturation, and the second is a legislative and increased understanding and acceptance with regulatory authorities. As regenerative therapies, PS cells derived tissues are neither recognized as solid organ transplants nor as pharmaceuticals. A novel legislative group of therapeutics had to be created to regulate these new era biological treatments, known as advanced therapy medicinal products (ATMPs)[74]. A tailored set of regulations, much more demanding to meet, has been set up, reflecting the complexity of the therapies.

As it comes to technological hurdles PS cells has brought many innovative techniques to the table, iPS cells in particular. As mentioned previously, ES cell-derived therapies have progressed further, partly because ES cells was discovered almost a decade prior and partly because ES cells do not require reprogramming to reach a state of pluripotency. The cons of ES cells include the ethical datable source, the inherent difficulty in donor screening for Human leukocyte antigen (HLA)[75] and the derivation of autologous transplants is not possible.

Due to the rapid emergence of the field, many technologies required to derive cells are still covered by patents. Patents can be vital for projects to securing funding but can also can limit developments when rights to key technology is kept exclusive. Freedom to operate allows academic research but can block commercial projects such as ATMP development. Own experience indicates that some holders of intellectual property (IP) are more permissive than others when it comes to licensing their technology for commercial enterprise. Licensing the use of the Yamanaka factors from academia Japan has been obtain by some and similarly the use of the Sendai vector technology. Reprogramming with certain mRNA kits is more complex. The more complicated IP landscape for iPS cell derivation, and the lower degree of IP protected technologies required for ES cell derivation, could, in the short term, favor the use of ES cells where legislation is permissive of their use.

2.1.1 Technologies for the Reprograming of Somatic Cells into iPS Cells

The process of reprogramming has been of concern as it necessitates the introduction of genes supporting the limitless proliferation of cells. Transplantations of undifferentiated pluripotent stem cells into immunosuppressed animal models leads to teratoma formation[12, 60]. However, there is no therapeutically potential of directly injecting PS cells, the concern has been that the graft could harbor small subsets of undifferentiated PS cells[76]. In this case both ES and iPS cells are equal, but with iPS cells there is also the added apprehension regarding the reactivation of integrated vectors could lead to tumor formation[77]. Therefore, it is essential that the vectors used to deliver the reprogramming factors are transient in nature, footprint free and carry no risk of integration into the genome.

This first iPS cell lines where derived by integrating retroviral vectors[60]. Retroviral vectors are associated with several issues, including, reactivation of pluripotency factors[77], insertional mutagenesis[25] and interference with the differentiation of iPS cells[78]. As the field evolved, so did the reprogramming methods available. A leap forward was taken with the introduction of reprogramming via episomal transfer vectors[78]. While this method offers transient expression of factors, the DNA based nature of this technique means the unintended integration of vectors by homologous recombination is possible. This has, as of yet, not disqualified therapies based on episomal vectors to spearhead iPS cell derived cell therapy developments. Although, tedious and costly screening for integration of reprogramming factors has been required before clinical use.[65, 79] Also, cells require prolonged passaging in vitro to shed the vectors[80]. Prolonged iPS cell culture risk introducing and selecting for genetic and epigenetic abnormalities[81-83]. Derivation of lines using episomal vectors is further complicated by the very low reprogramming efficiency of about 0,0013%[84].

Development of RNA based non-integrating vector systems would eliminate the need to screen for transgene integration as vector integration through genomic recombination would not be possible[85]. Sendai viral vectors was introduced as an alternative, resulting in strong yet transient expression of factors without having a DNA-phase, deriving iPS with high efficiency of >1%[86]. Reprogramming vectors delivered by Sendai virus require extensive passaging to ensure shedding vectors (Figure 3)[26].

A new generation of mRNA based[85], recombinant human protein[87] and small molecule compounds[88] offer more transient expression of factors and allow quicker derivation of lines, while reducing the extensive post-derivation safety testing. Difficulty has surrounded the development of these methods. Protein reprogramming technology remain costly and inefficient[89] while small molecules has, to date, not been proven to work in humans[90]. Of these methods, only mRNA based reprogramming has developed to become an established form of reprogramming[84].

The strength of mRNA vectors is the highly transient nature of the system. Within 42 hours of vectors administration the exogenic protein levels have returned to baseline[85]. mRNA offers a footprint free method of reprogramming, abolishing the need to screen for transgene integration or for prolonged passaging, and eliminate the use of virus while offering a good efficiency. So far, use of mRNA reprogramming have been limited to adherent cell types,

such as fibroblasts, no account of reprogramming of peripheral blood mononuclear cells (PBMC) cells could be discovered. The difficulty in transfecting PBMCs is because of high cytotoxic response to the mRNAs in the cell type[91] Blood samples can still be used for reprogramming but depend on the establishment of endothelial progenitor cells[91].

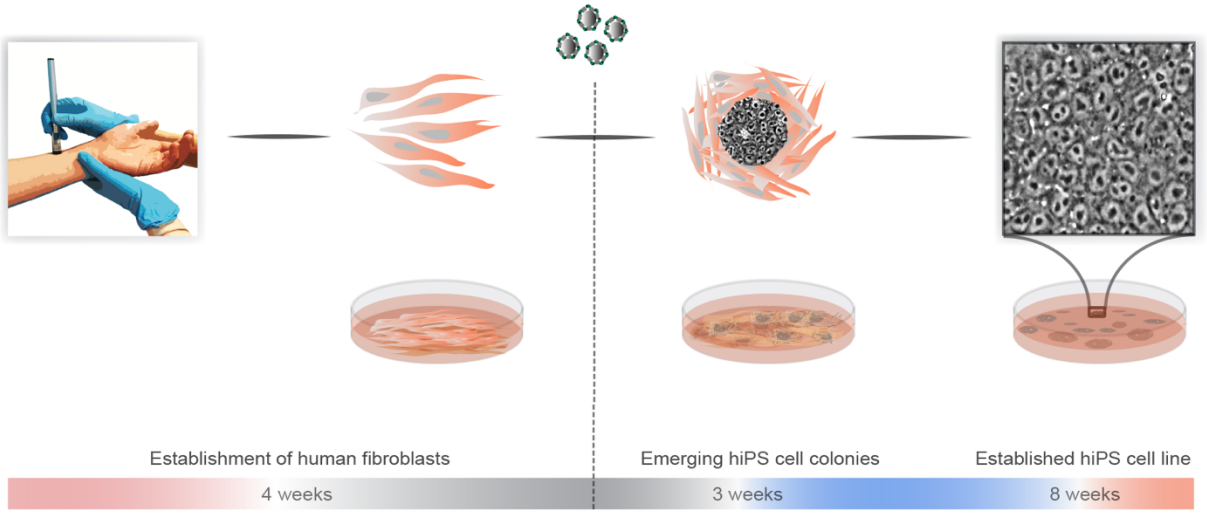

Figure 3. The isolation and reprogramming of human dermal fibroblasts. The timeline applies to Sendai viral vector reprogramming. Experiences from own lab indicate mRNA reprogramming can shorten derivation by up to 5 weeks. Adapted from Uhlin, Marin Navarro et al, 2017[2].

2.1.2 Development of Clinically Relevant Cell Culture Systems

The effect of the culture system on the cells cannot be overstated. A plethora of factors contribute to determining cell fates including, mechanical forces, cell contact [92] culture matrix, paracrine factors[93], and the potential presence of cell subpopulations[44, 94]. Balancing these factors is fundamental to achieve the desired result. Replacing any culture component can lead to differences in the end products. Two recent studies showed preclinical failure of cell therapies when research grade components had been substituted for clinical reagents and manufacturing subjected to good manufacturing practice (GMP)[95, 96]. It is therefore essential to establish a robust clinically relevant protocol as early as possible as clinical adaptation often affect the cell product.

With the imminent commencement of several of iPS cell based therapies clinical trials, demands put on the reproducibility, standardization of batches, and proof-of-safety are vital to address[97]. Ideally, cell culture systems aimed at clinical use should if possible exclude products of animal origin and where possible only use defined components[98]. A multitude of arguments have been put forward against of xenogeneic and undefined culture conditions including the risk of microbial contamination, variability of the final product and immunogenicity of derived cells[99, 100]. Transfer of non-human immunogenic siliac acids has been confirmed to occur from xenogeneic components to stem cells in culture[101].

Early culture systems were dependent on coculture with fibroblasts feeder cells layers[12, 102] or undefined matrixes such as Matrigel[103] and culture with media often enriched by fetal

bovine serum (FBS)[12, 60]. Batch-to-batch variability is associated with these components leading to variations in the final product[100]. Development of defined cell culture matrixes for instance Laminin-521[104]or vitronectin[105] and the refinement of cell culture medias such as Essential 8[106] allows for defined cultures with reduced variability and reproducibility of protocols.

The implications for regulatory approval of cells derived using animal or undefined components for the production of cell for clinical use has so far been handled on a case to case basis by "risk-based approach". The general notion is that if better alternatives are available those should be used. The inclusion of such components has carried with it strict demands of proving their use is safe and result in sufficient quality[107] This often result in laborious work, or the development and implementations of various assays into the quality control regiment. In addition to these technical hurdles, the use of FBS and coculture with feeder cells provide major obstacles for the scale-up of production and thereby hindering the emergence of novel therapies[108, 109]. Taken together, xenogeneic and undefined components raise concern of variability and safety and should be excluded where possible.

2.2 ESTABLISHMENT OF COHERENT REGULATION OF ATMPS

With many of the technological hurdles considerably flattened by technological maturation, the establishment of internationally harmonized legislations is called for. Such legislation should be capable of safeguarding patient interests without smothering progress while allowing international approval of therapies. Today, there are no universal regulations in the field regarding ATMPs. Assessments are done by the individual regulatory authorities. However, there are ongoing efforts of harmonization in-between authorities, primarily the European medicines agency (EMA), the Food and drug administration (FDA), the Japanese and the Canadian authorities[110]. This text will focus on the regulations by the EMA, responsible for issuing market approval in the EU and the Swedish medicinal products agency, responsible for approval of clinical trials conducted in Sweden. The nearest thing to universal legislation available is the guidelines presented by the international society for stem cells research (ISSCR). The ISSCR regularly presents guidelines on stem cell research and clinical translation, commenting on preferred technical practices, but also ethical and policy changes as the field develops[98].

The international conference on harmonization of good clinical practice (ICH-GCP) was launched as an attempt by the regulatory authorities in European Union, United States and Japan to "provide a unified standard to facilitate the mutual acceptance of clinical data by the regulatory authorities in these jurisdictions". According to these guidelines any investigational product must be produced under GMP[111]. Solid organ transplantations are not subordinate to these rules, however, PS cell-derived therapies are classified by the EMA as ATMPs which requires manufacturing under strict GMP[74] The purpose of which is defined by both the EMA and the FDA as, the ability of manufacturing process to meet pre-set product criteria through control, donor testing, traceability, documentation of manufacturing and development and later the quality control of the product. The rules have been developed to safeguard consistent quality and guarantee the safety of the products in clinical use[97].

Neither good clinical practice (GCP) nor GMP does forbid the use of animal or undefined products unless better options are available, but requires meticulous risk assessment for their inclusion[112]. The legislations are regularly updated and adjusted for recent developments and provide increasingly specific technical requirements and standards[113]. These legislations stress the importance that the safety of the product must be evaluated. The safety of PS cell derived products needs to be fully addressed and demonstrated by preclinical data including chromosomal stability, biodistribution, cell identity, sterility, mutations in oncogenes and housekeeping genes as well as genes involved with cell function. Currently, there is little agreement of what standards should be used or even how the data should be interpreted.[71] Clearer standards would facilitate developments and should be set on a multinational scale.

2.2.1 Derivation of Clinically Relevant cGMP Compliant iPS Cell Lines

The first pluripotent stem cells used to derive tissues for the first clinical trials were not up to the standards described above. Many of the lines were derived using xenogeneic substances, on feeder cells, in research grade facilities, with insufficient consent and then been subject for post- derivation classification as cGMP compliant by additional testing[114]. Such inverse qualification is unlikely to be recognized in the future since alternative derivation methods have since been developed.

To date, only five groups have published the successful derivation of fully GMP compliant iPS cell lines[79, 115-118] highlighting the apparent difficulty of such an endeavor (Table 1). Even so, the IPS cell line going into the clinics in the first ever trial[65], was not manufactured under GMP but good laboratory practice (GLP), and subject to a series of exemptions allowing the ground-breaking use in humans, in a bid by the Japanese government to spearhead developments in regenerative medicine[119, 120]. Attempts to circumvent the regulations by converting research grade lines to GMP-grade by introducing them to a different culture system and meticulous quality control testing, deriving what is referred to as "putatively GMP compliant" has been suggested as a faster and less costly way to derive lines[121]. The GMP compliant cell lines published by Baghbaderani BA et al. by the efforts of the Lonza corporation published a protocol not fully living up to the goal of producing cells in a xeno-free and defined manor, using both xenogeneic products and undefined components[79]. Wiley et al, were able to fully exclude xenogeneic products from their iPS reprogramming and maintenance process, however the authors highlight the dependency on human serum for the culture of fibroblasts as a drawback[116, 122]. Haase et al. recently published a fully xeno-free and defined derivation of cGMP compliant lines by sendai virus reprograming of hematopoietic stem cells from peripheral blood[118, 123]. Quite an achievement, however, substituting the sendai virus mediated reprogramming for a non-viral vector mRNA vectors could shorten production the production time and boost the clinical relevance even further[85]. The manufacture of GMP compliant lines are clear signs that the technical developments are approaching maturity. What remains now, is to ensure the use of GMP manufactured lines going forward and to derive cell therapy products of equal standard.

PS Cell type	Author	Xeno-free	Defined	Starting material	Reprogramming Vector	GMP Compliance	Year Published
ES	Thomson[12]	No	No	Embryo	N/A	No	1998
iPS	Kamao et al[65, 124]	No	No	Dermal Fibroblasts	Episomal	No	2014
iPS	Baghbaderani et al[79]	No	No	Cord blood	Episomal	Yes	2015
iPS	Wang et al[115]*	Yes	No	Foreskin fibroblasts	Sendai	Yes	2015
iPS	Wiley et al[122]	Yes	No	Foreskin fibroblasts	Sendai	Yes	2017
iPS	Haase et al[118]	Yes	Yes	Dermal fibroblasts	Sendai	Yes	2019

Table 1. Clinically relevant PS cells. The first ES cell line and the first iPS cell line used in clinics (top of the table), and four of the five published iPS cell lines derived under GMP compliance. Note, the ES cell line and the first iPS line to reach clinical use was not produce under GMP. *not necessary under the ICH definition of GMP.

2.2.2 Considerations for Development of Cell Therapies from Human Pluripotent Stem Cells

To widen the perspectives, the objective is not solely to provide proof of concept but for the development of novel therapies to benefit the public. Academic scientists must consider the later stages of development or risk developing functioning therapies which can never be commercially viable and hence never reach the intended patients[125]. To successfully develop a cell therapy, it is necessary to adopt a backwards approach and consider three key points which are vital to address if a therapy is to have a chance of becoming successful. These are characterization, scale-up and cost of goods[126].

Characterization which is essential for safety, efficacy and to ensure comparability when the protocol is inevitable adjusted for scale up or when components are substituted. For scale up to be possible, it is important to minimize time consuming manual processes and choose culture system that allows to be expanded to larger size or translated into bioreactors. Every part of the chosen manufacturing process will be reflected in the cost of the final product. The most imperative to consider here is if the therapy can be allogenic or autologous, and if cryopreservation will be possible. Cryopreservation can vastly extend shelf life and enable storage and distribution of doses. The first in human trial for ES cells derived therapy and iPS cell therapy provide widely different approaches to some of these concepts.

2.2.3 The World's First Pluripotent cell Derived Cell Therapy in Clinical Trials

The Geron corporation developed the world's first pluripotent cell derived cell therapy, consisting of oligodendrocyte progenitor cell therapy for subacute SCI and progressed to phase I clinical trials[127]. A total of five patients was grafted before a premature shutdown of the trail. According to all indications the trail progressed well with no indications of serious adverse events and termination was instead due to economic considerations[128].

The protocol used allogenic, cryopreserved doses of oligodendrocyte progenitor to which a thorough characterization protocol was applied. The thorough characterization accelerated the positive risk benefit assessment issued by the FDA contributing to the pioneering achievement several years ahead of its time.[129] A very elegant set up which could with relative ease be scaled to meet future market demands and distributed.

Many ethical and other criticism has rightfully been raised against the handling of the trail, choose of condition, patients and premature cancellation[128]. However, developments are still ongoing under Asterias that acquired Geron in 2012, and Phase I/IIa clinical trials with 25 participants was resumed in 2014 and concluded in 2018[130] with no serious adverse events reported and 95% of subjects reporting improved motor function[131].

A possible benefit with using progenitor cells is that they are not necessary disease specific, with a wide differentiation potential more diseases can be treated with the same drug product. Dopaminergic neurons are specific to Parkinson's disease while OPCs, MSCs, NS cells or NES cells are not. Safety data can be used for other application of the same drug an facilitate cheaper and faster developments. OPCs could hypothetically also have uses in MS and white matter stroke[131].

2.2.4 iPS Derived RPE Tissue for Age Related Macular Degeneration

If the protocol of the first ES cell trail can be described as elegant and scalable the opposite must be said for the first iPS cell trial were little considerations was taken. A tissue transplant of non-cryopreserveable Retinal pigment epithelium (RPE) cell sheet derived from patient specific autologous cells as a therapy for age related macular degeneration[65]. Other groups has estimated that deriving autologous iPS cell-derived NS cells takes at least 6 moths excluding safety testing[132], the time required in the RPE cells is likely somewhat similar. This, paired with the cost of establishing cGMP compliant iPS lines were derivation and safety testing of the cell line it most likely in the area of 165 000 €[133]. A staggering expense for the derivation of a cell line intended for only one patient. Dose delivery of the RPE sheets also require more intricate surgery compared to the injectable cell suspension as was the case with Geron's OPCs.

2.2.5 Goals of the Sponsor

These apparent polar opposites in trial design is reflected by the different goals of the trial sponsor. The ambitions in the iPS cell derived RPE cell trial was rather academic and aimed to provide proof-of-concept rather than to develop a blockbuster therapy. Although the

approach taken does minimize the commercial feasibility it serves the function of laying the foundation for which new bold projects can take shape. Since the first patient was transplanted in 2014 a new direction has been taken by Takahashi's group which recently transplanted iPS derived RPE cells which are allogenic and HLA-matched to the recipient. This change vastly bolstered the possibility of creating a cell therapy reaching more patients.[132]

2.3 AVOIDING GRAFT REJECTION IN IPS CELL DERIVED THERAPIES

2.3.1 Autologous Grafts

ATMPs are indeed a beast of its own. Unlike small molecule-based pharmaceuticals, cell therapies risk destruction by the immune system. As with all types of transplantation, PS cell derived grafts will be subjected to recognition and destruction by the host adaptive immune response[134]. The holy grail of iPS cell would be to grow any transplant from the recipients own cells, potentially eliminating the need for immunosuppressive therapy. Experimental comparisons of autologous and allogenic iPS graft transplants suggest that autologous grafts elicit low or none-existent adaptive immune response, displaying better graft survival even in the absence of immunosuppressive therapy in non-human primates[135]. Although, autologous therapies may be ideal, they are today, unfeasible on a large scale. Both the derivation cost, and time required for clinically relevant, GMP-compliant iPS-line is beyond the scope of large scale therapies[136]. Also, certain conditions may arise because of genetic traits in the patient. In these cases, autologous cells could be unsuitable since the derived cells would recapitulate the phenotype[137].

2.3.2 Matching of Donor and Recipient in the Stem Cell Era

iPS cell technology allows for screening and donor selection on the basis of health status, HLA-type or blood type. The advantage of HLA-matched transplants lies in reducing immunogenicity in host and limiting immunosuppressive regimes. Essential for matching to be cost effective and practically feasible is to limit the size of the cell bank required by maximizing the population coverage and boosting the interchangeability in-between genetically distant populations.

The notion of establishing an allogenic PS cell bank, HLA-matched to the population was first suggested by Taylor et al in 2005, even prior to the derivation of the first iPS cell lines. The authors suggested the derivation of 150 ES lines at random could provide HLA-A, HLA-B and HLA-DR match for 18,5% of the UK population[138]. With the dawn of iPS cell-technology it became possible to screen for donors of the most common HLA-haplotypes prior to cell derivation[139]. Taylor et al returned with a publication in 2012 where they searched Bone marrow worldwide registry for potential donors for iPS cell derivation. By using a strategy of finding rare donors homozygous for HLA-A, HLA-B and HLA-DR, a strategy could be formulated and a bank of just 10 cell lines providing a match for 37,7% of the UK population[75]. Previously, Okita et all presented a strategy where 140 homozygous lines, selected from 160 000 screened individuals would provide match for 90,7% of the Japanese population[140].

Strategies on stem cell banks have mostly been on the national level, population heterogeneity varies largely in-between people of different ancestries and does not align with national borders. Studies on the diverse American population has shown that Americans of European ancestry is a most homogeneous group, with a very high degree of interchangeability, while largely different from African Americans and Asian Americans[141]. The most common haplotype identified by Okita et al, providing a match for 8,5% of the Japanese population is not even present in the list of the top 236 lines presented by Taylor et al[75]. This highlights the need for creating different banks optimized for different ancestries, establishing common banks across national borders, and call for Haplotype sharing in-between banks in the ever-globalizing world of today.

Previous strategies have often limited their strategies to only providing a limited match to selected HLA alleles, contending with only partial matching. Optimal match for transplantations should take into account all alleles of HLA class I and HLA class II loci, exclusively select donors with blood group 0, preferably use female donors to avoid Y linked minor histocompatibility antigens[136]. However, actualizing even the more limited banks, similar to those suggested above represents an enormous effort.

2.3.3 Genome Editing of HLA-Molecules

Genome editing approaches such as CRISPR-Cas9 can with relative ease be applied in stem cells lines and target certain HLA-molecules increasing population match achieved[142]. One opportune target for gene editing is the Class II trans activator (CIITA) which is required for the transcription of HLA class II molecules[143] or the Beta-2-Microglobulin (B2M) molecule required for HLA class I cell surface expression[144].

2.3.4 Universal Cell Lines

Complete knockout of both these genes has been suggested to create non-immunogenic universal cell lines hypothetically tolerated in all recipients[145] However, natural killer cells are specifically tuned to recognize the missing self-response and this strategy require the knock in of an exogenous HLA-E-B2M protein[145]. Potentially a very useful way of circumventing the demands of matching cells but the HLA molecules do serve important immune functions, important to fight infections or malignancies. The latter is especially troubling given the concerns of tumorgenicity of pluripotent cells.

2.3.5 HLA-Retention Strategies

A recent study suggested the retention of single HLA-C molecules. The retained molecule would deter Natural killer (NK) cells, preserve a degree of antigen presentation capability while minimizing the cell lines required for matching a majority of the Japanese population[146]. Essentially allowing the creation of a limited cell bank while partly conserving the important functions of the immune system.

2.3.6 Suicide Switches

Introduction of inducible "suicide genes" are sometimes discussed as a failsafe to prevent graft overgrowth, teratoma formation or malignant transformation[147] Such switches are placed under the control of small molecules and induce selective apoptosis of the grafted cells[148]. Nevertheless, the inclusion of such genes comes with risks of its own and are delivered by adeno-associated virus or integrating viral vector which will require additional regulatory oversight[132].

Genome editing approaches are capable of improving cell therapies but does come with concerns themselves, appropriate measures to ensure no off -target effects or other safety concerns will need to be answered as prescribed by the risk-based approach. Interestingly, the cancer therapy field may offer rare precedent were gene edited chimeric antigen receptor T- (CAR-T) cell therapies has advanced far enough to receive market approval[149].

3 PLURIPOTENT CELL DERIVED CELL THERAPIES

3.1 DERIVATION OF CELL TYPES RELEVANT FOR REGENERATIVE THERAPIES

Many cell types with grand potential for regenerative medicine have been successfully derived and tested preclinically. Including cardiomyocytes for myocardial infarction[150] , RPE cells for macular degeneration[151], ß-cells in insulin dependent diabetes[152], dopaminergic neurons for Parkinson's disease[134], neural stem (NS) cells [153]and neuroepithelial stem (NES) cells[154] for SCI (Figure 4).

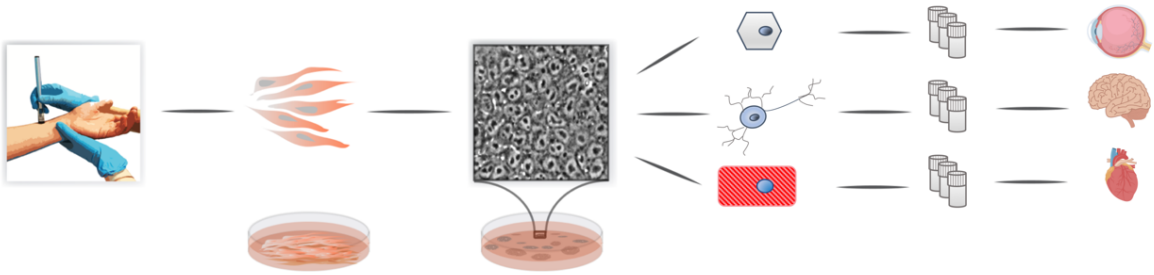

***Figure* 4. From dermal biopsy to iPS cells and further on to differentiated disease relevant cell types.** Cell culture require advanced cell laborites and highly skilled labor. Developing efficient freezing thawing protocol, means cells could be supplied to clinics in cryovials, thawed at convince in the operating theater and injected with minimal manipulation. The dermal biopsy donor can be the same as the recipient of the cell therapy as is the case in autologous cell therapy, which results in an optimal immunological match. Alternatively, the donor can be allogenic which enables the derivation of large-scale cell therapy batches. Adapted from Uhlin, Marin Navarro et al, 2017[2].

3.1.1 Differentiating Pluripotent Stem Cells into Disease Relevant Cell Types of the Central Nervous System (CNS)

Efficient protocols for the neural induction of PS cells were reported over a decade ago and enabled the directed differentiation into disease relevant neural cell types[155]. iPS cells can be terminally differentiated to post mitotic neurons or perhaps more promising captured along the path of differentiation as immature, proliferating neural progenitor cells. Grafting of immature progenitor have shown to improve integration while retaining the capacity to proliferate and differentiate after transplant[156]. The capture of several different types of expandable neural progenitor cells with different temporal and spatial properties has been reported upon neural induction of PS cells[157].

3.1.2 Pluripotent Cell Derived Neuroepithelial Stem Cells: a Candidate for Repair of the CNS

NES cells are a type of expandable multipotent neural stem cell type capable of differentiating into astrocytes[158], oligodendrocytes and neurons[159]. NES cells can either be derived from pluripotent cells[159] or from fetal hindbrain corresponding to gestational week 5-7 week[160].

The trilineage differentiation potential, high proliferative capacity and the ability to integrate into the mammalian CNS makes NES cells of interest for cell therapy of the CNS[157]. NES cells are primary neurogenic and differentiate at ratios of about 90% to TUJ1 positive neuoranal cells and 10% giving rise to glia[159]. Preclinical investigation of NES has so far focused on SCI where grafted animals have displayed cell integration, differentiation and some degree of functional recovery[154, 161]. NES cells can also serve as intermediate in the derivation of astrocytes[158] or dopaminergic neurons which have potential uses in regenerative medicine[162].

A similar cell type, neural stem (NS) cells can be derived from later developmental origin around gestational week 8. NS cells exhibit a more gliogenic differentiation profile[163] and differentiate at a ratio of about 50%-50% to neurons and glia[164]. Elegant studies have developed well characterized, cryopreserved cell therapy from fetal NS cells which entered clinical trials for amyotrophic lateral sclerosis in 2012[165]. The proof of safety from the amyotrophic lateral sclerosis trials allowed the same fetal derived NS cell line to be used in clinical Phase I trials for SCI in 2016. Four patients were grafted with cryopreserved NS cell doses with no serious adverse events reported and with some degree of functional recovery in two of four patients[166]. This provides another example of an ATMP with the potential of treating several indications were the safety dossier can be used in support of new applications.

The fetal origin of these cells comes with many ethical issues and are inevitable a limited resource. Nonetheless, fetal cells have once again a come to pioneer cell therapies developments and do so because they circumvent the safety concerns associated with PS cells. Another reason for fetal cell grafts predating the use of pluripotent derived cells is the time required to develop new therapies. When the fetal NS cell line used in the trial was derived in 1996 it preceded the derivation of the first human ES cell lines and the first human

iPS cells by more than a decade. These advances made by fetal cell grafts serve as excellent proof of concept and pluripotent derived NS cells have now been approved for clinical trials in Japan[132].

Human iPS derived NES cells has been shown to have a comparable therapeutic potential to that of fetal spinal cord NS in mice[154]. Recent unpublished data indicate that NES cells have improved preclinical effect over NS cells in SCI associated traumatic syringomyelia[167]. NES cells also grow faster, with doubling time of 24-30 hours[159] compared to 2-3 days for NS-cells[163], a useful characteristic for the large scale derivation of cell therapy doses.

3.2 FUTURE APPLICATIONS

It is important to keep in mind the tremendous progress in the field in the decade since first discovery when considering the future. What we deem unfeasible today many well be reality in a few years. The pace of developments during this PhD project has been extraordinary. An open mindset and innovative approach will be essential to navigate in this environment.

It is encouraging to see the first clinical trials involving PS cells reporting no serious adverse events, and to see the first patients receiving therapies derived from iPS cells. It remains important to remember that this initial success can rapidly be lost if negative safety indications appear. The field of gene therapy is often exemplified, which was set back several years because of the emergence adverse events[168]. Proceeding with caution may prove the fastest way forward.

As therapies are beginning to move past phase I and phase II clinical trials, the scale of manufacture will move from a flexible scientific scale to an industrial scale. With the scale increase and the implementation of automation, it is likely that costs of goods can radically be decreased in the near future. The technology supporting the scale up from small manual culture vessels to massive bioreactors has already been demonstrated with PS cells growing either in suspension or on microcarriers[169]. Rough estimates on the requirements of commercial manufacture puts the need of around 10^{11}-10^{14} cells per year of average products which would equate the annual output of one 200 - 2000 liter bioreactor[170].

The New York Stem Cell foundation reported the development of a modular robotic platform capable of deriving research grade iPS cells from dermal biopsies. The platform was also capable of some basic characterization, with minimal manual intervention. Not only a promising alternative to manual derivation because of cost reasons, it was also validated that the lines produced displayed less variation compared to manually manufactured lines[171]. The automated system was reported to reduce reagent cost 5-6-fold[171]. Reprogramming by microfluidic chips on a microscale has reported successful reprogramming with a 100-fold reagents saving compared to standard 6 well reprogramming. Such microfluidic chips enabled 32 parallel reprogramming's simultaneously in an automated fashion[172].

Although autologous cells may be unfeasible for widespread use today, it might not be so in the future. Just like high throughput screening revolutionized drug testing we may soon enter a future where high throughput cell derivation dominates. Robotics also increase the distance

of humans from cultures, humans often being a source of infectious organisms threatening cultures. Other benefits include robots being more consistent and can implement automatic feeding regimes based on sensors reading, changing culture components when needed rather than on predetermined intervals[169].

To conclude, iPS cells has the potential revolutionize medicine and developments in the last decade has went from discovery up to the threshold of in human applications. Much work remains to be done especially with regards to the legislative development and harmonization. Deriving the PS cell is part of the process. Equal considerations must be endowed the development of the specialized cell grafts transplanted.

When can we expect to see cell therapies available to the public? The first iPS cell derived therapy product to conclude phase I trial was an MSC therapy in Graft vs. host disease, showing no serious adverse events and provided indications of efficacy[67, 173] Estimates suggest PS cell derived therapies for macular degeneration within 2-5 years, diabetes is currently in phase I/II and will take longer, heart disease is in Phase I. SCI around 5 years. For the first iPS cell applications in eye disease is pioneering[71] but trials have been approved for heart failure, Parkinson's disease, SCI and are scheduled to commence imminently[132].

Aims of this thesis

The overall aim was to investigate the potential of iPS cells to be used in regenerative medicine.

A few key projects were identified

- Developing clinically relevant, xeno-free and defined protocols for the derivation of iPS cells
- Establish a process for the cGMP compliant manufacture of iPS cells
- Explore the feasibility of a HLA-mathed iPS cell bank of clinically relevant cells matched to the majority of the population
- Develop clinically relevant protocol for the neural induction and capture of NES cells derived from iPS cells
- Examine the potential of using NES cells to develop an ATMP for Spinal cord injury

4 RESULTS AND DISCUSSION

The main focus of this project was to derive clinically relevant iPS cells. By our definition, this included: elimination of all animal components, using only defined-reagents, and reprogramming by a non-integrating footprint-free reprogramming system. Once a robust clinically relevant process had been developed, spanning from biopsy collection to iPS cell cryopreservation, GMP compliant manufacture would be attempted.

There is no clinical utility of iPS cells alone, therefore it was decided to investigate the therapeutic potential of NES cells in SCI. The NES cell derivation protocol should preferably also conform to our definition of clinical relevance and if possible be derived using clinically relevant iPS cells if these could be developed.

To avoid investing resources into the development functional but commercially unviable ATMP, we decided to take a backwards approach and factor in the possibility of characterization, scale up, cost of goods in the early studies of our ATMP. As a part of this effort we also decided to investigate alternatives to using patient specific iPS cell lines and cryopreservation of cell therapy doses.

Paper I outlines the derivation of two research grade iPS cell lines from skin biopsies intended for the use as controls in disease modeling projects. In this paper we use a method we developed to reprogram fibroblasts by non-integrating sendai viral vector which were maintained as iPS cells in a fully xeno-free and defined culture system. **Paper II** further sough to define and describe our process in minute detail. This was essential for the upcoming technology transfer to the GMP facility and the authoring of standard operating procedures required for the GMP compliant manufacturing process.

Fibroblast derivation was still performed using fetal bovine serum and non-recombinant enzymes in the treatment of the dermal biopsy. To derive clinically relevant cells by our definition we sought to fully eliminate xenogeneic and undefined products from all steps of our process. The developed method was with the exception of the biopsy preparation and fibroblast culture suitable. To find an alternative to media containing FBS in the culture of fibroblast we tested a series of commercially available media formulations. We were made aware that culturing fibroblasts is permissive as long as the primary culture has been established with serum but found the removal of serum in the early process yielded few cells with very limited proliferation. We set out to enhance the fibroblasts yield from our biopsy preparation by improving both conditions and including additional factors which improved the outgrowth from biopsies in a fully recombinant and defined method. We had also begun testing of mRNA mediated reprogramming in the laboratory, a method ideal for derivation of cells for clinical applications, however complicated to master as the mRNAs give rise to strong innate immune response in the reprogrammed cells.

To facilitate the future regulatory approval of our GMP manufactured cell lines, we were required to scrutinize all reagents used in our process and tested alternative reagents were

necessary. Consultations with the Swedish medical products agency proved an invaluable resource to develop a mutual understanding for the requirements for eventual approval.
For **manuscript III,** we had built on the previous iPS expertise in the previous papers and developed a similarly robust protocol using mRNA reprogramming and only clinically suitable reagents. Several clinically relevant lines were derived by this method to ensure the robustness of the method and to determine the process in detail before we could attempt to transfer our method to the GMP manufacturing facility.

Extensive documentations was authored and the method adapted to GMP before manufacture could commence resulting in the cGMP compliant iPS cell line KICRi-001-A also known as clone #10.

Manuscript V embodied what been learned along the project when it came to the considerations for developing an ATMP. A backwards approach was taken with the focus on creating a cell therapy product which could be scaled, characterized at a minimal cost of goods. Important for future regulatory approval was to develop a meticulous characterization program that could be performed on each individual batch rather than on the cell lines. Such characterization would require some form of preservation strategy since the quality controls required would take far longer than shelf-life of live dissociated cells. Considerable effort was spent developing a cryopreservation regime of NES cell therapy doses to develop an off-the-shelf therapy which could be injected directly without the need of culturing cells “in clinic”. Cryopreservation is also important in keeping cost of goods to a minimum, since large banks of doses can be manufactured, stored and distributed.

Developing ATMPs is very costly and depending on autologous cell source was not consider a feasible alternative. Considering either a non-matched allogenic or a therapy with HLA-matched cell lines discussed in **Manuscript IV.** We judged the previous strategies presented was unfeasible, as to many lines were required. First, we attempted to demonstrate the unfeasibility of this approach by estimating the population screen required to identify donors and the cost of realizing such a strategy. Cost and screen were factors we had found being insufficiently explored in previous publications. We then devise a strategy which we deemed viable. The use of genome editing was found to greatly reduce cost, increase population match and interchangeability in-between distant populations.

Going forward towards the future with a vision of cryopreserved cell banks of disease relevant cell therapy products. Either by a one line fits all approach using universal cell lines or possibly by each product consisting of a dozen of HLA-retained cell lines. Such cell therapies could be stored in hospital pharmacies around the world and with a fairly simple procedure be injected to regenerate injuries. Few fields of science are likely to be as exciting as the upcoming decade of regenerative medicine. I consider myself fortunate to have had the great opportunity to partake in this exhilarating field of research.

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